Induction of Heavy-Metal-Transporting CPX-Type ATPases during Acid Adaptation in *Lactobacillus bulgaricus*[▽]

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Lactobacillus bulgaricus **is a lactic acid bacteria (LAB) that, through the production of lactic acid, gradually acidifies its environment during growth. In the course of this process,** *L. bulgaricus* **acquires an improved tolerance to acidity. A survey of the recently established genome sequence shows that this bacterium possesses few of the pH control functions that have been described in other LAB and raises the question of what other mechanisms could be involved in its adaptation to the decreasing environmental pH. In some bacteria other than LAB, ion transport systems have been implicated in acid adaptation. We therefore studied the expression of this type of transport system during acid adaptation in** *L. bulgaricus* **by reverse transcription and real-time quantitative PCR and mapped transcription start sites. Intriguingly, the most significantly induced were three ATPases carrying the CPX signature of heavy-metal transporters. Protein homology and the presence of a conserved sequence motif in the promoter regions of the genes encoding these proteins strongly suggest that they are involved in copper homeostasis. Induction of this system is thought to assist in avoiding indirect damage that could result from medium acidification.**

Lactobacillus bulgaricus belongs to the lactic acid bacteria (LAB), a taxonomically heterogeneous group of bacteria characterized by the production of lactic acid and concomitant acidification of their environment. Within this group, *L. bulgaricus* is one of the economically most important representatives as a result of its worldwide use in the production of yogurt, the product of milk fermentation by *L. bulgaricus* and *Streptococcus thermophilus*.

Traditionally, yogurt consumption has been credited with having beneficial effects on health (2), and some of these effects have been shown to depend on the presence of live bacteria (45, 54). In addition, depending on the country, national laws may require yogurt to contain at least 10 million live bacteria per gram at the time of marketing. This makes bacterial survival during fermentation, product handling, and throughout shelf life an important issue. An important property of yogurt that affects bacterial survival is its acidity. During yogurt fermentation, lactic acid production causes the pH to fall to about 4.2, down from an initial value of 6.6 to 6.8 in milk (http://www.fao.org/).

Functions that have been implicated in acid adaptation in LAB include those involved in changes in the cell envelope and repair of DNA and protein damage that often play a role in the response to other stress factors as well (13, 19, 53). In addition, mechanisms that play an active role in the control of cellular pH and proton motive force have been described. These generally include H^+-expelling proteins or protein complexes, notably the H^+ ATPase, and enzymes involved in the production of alkaline compounds.

A less studied group of proteins that may play a role in acid adaptation consists of ion transport systems. A chloride channel (23) and a K^+ uptake system (15) have been implicated in acid resistance in *Escherichia coli* and *Streptococcus mutans*, respectively, and assigned a role in the equilibration of electric charges when the H^+ ATPase or other systems are proceeding to a net efflux of protons.

Here we address the question of how *L. bulgaricus* gradually adapts to the low pH it creates in the culture medium during growth and escapes the fate of many late spoilage bacteria that are inhibited or killed when suddenly confronted with the hostile acidic environment. We present an inventory of acid adaptation mechanisms known from other LAB and found in the genome of *L. bulgaricus* and a study of the expression of ion transport systems during acid adaptation.

MATERIALS AND METHODS

Bacterial strain and growth conditions. The bacterial strain used throughout this study is *L. bulgaricus* ATCC 11842, the type strain of the species, obtained from the American Type Culture Collection. *L. bulgaricus* was cultivated at 42°C in chemically defined medium (MPL [12]) containing 5% lactose and 50 mM morpholinoethanesulfonic acid (pH 6.5). Additional vitamins (1 mg/liter cyanocobalamine, 5 mg/liter orotic acid, 5 mg/liter 2-deoxythymidine, 2.5 mg/liter DL-6,8-thioctic acid, and 5 mg/liter pyridoxamine) were added to this medium where indicated (MPLm), to improve growth. CFU were counted after plating on MRS agar medium (Difco Laboratories, Inc., Detroit, MI) and incubation for 42 h at 42°C under anaerobic conditions.

Determination of acid tolerance and adaptation. A single fresh colony was used to inoculate 1 ml of MPL, and the culture was incubated for 8 h at 42°C without shaking. The culture was then diluted 1,000-fold in 13 ml of prewarmed MPL medium, and incubation was continued overnight. The resulting culture (with an optical density at 600 nm [OD_{600}] of <0.2) was diluted to an OD_{600} of 0.05 with prewarmed MPL and incubated until the OD_{600} reached 0.2. To measure acid tolerance, cells from 1 ml were harvested by centrifugation at 13,000 × *g* (2 min at room temperature), and the pellet was resuspended in 1 ml of MPL adjusted to pH 4.0 with lactic acid. Immediately after resuspension and after 30 min at 42°C, serial dilutions were made in MRS broth, and aliquots were spread on MRS agar medium. Survival was calculated by dividing the number of CFU per ml after incubation at pH 4.0 by the number of CFU per ml immediately after resuspension.

For experimental acid adaptation, the culture with an OD_{600} of 0.2 was divided into two aliquots. For one aliquot (nonadapted sample), acid tolerance was

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TABLE 1. Primers used in this study

Gene no. in L. bulgaricus	QPCR primer		Transcriptional start mapping primer[b]
	F or R[a]	Sequence	
Ldb0162	F	AAGTGGACCCTGCCTTACCT	GCAGAAGATGACCGTTGTGA
	R	GCAGAAGATGACCGTTGTGA	TGGTCAAGCAGGTAATGTCGT
			AGACTCGATCGTGTGCCAGT
Ldb0219	F	TGTCTGCCTCTCCCTGAACT	
	R	TGGCATAGAAGGGGTTGATT	
Ldb0226	F	ACCTGCACGACTCCTGGTAT	AAGTATCCAGCTTCCGGTCA
	R	AAGTATCCAGCTTCCGGTCA	GCTTGCCGCTCTCTGACT
			CCATCAAGATCCCGACGA
Ldb0341	F	CACCGGGATCAAGAACATCT	
	R	TCAAGGCATCAGTCCAGTTG	
Ldb0426	F	ATGGCCGGTGGAAAAGTCTA	
	R	ATCATGGCAAAACGGATGAA	
Ldb0456	F	GACCTGCGTCTTTTGGTAGC	TGCTGGAGTAAGCCATGTTG
	R	TGCTGGAGTAAGCCATGTTG	CGCGTTCAAAAGCACAACTA
			AAGCGGGCAAACATTGAAC
Ldb0483	F	CCGGCCTCTTTATTTTCTCA	AGAAACTTGCCGATCCAGTC
	R	AGAAACTTGCCGATCCAGTC	CTGGGCAAAAGAGCTGAGAT
			AGTAGCTGTACTTGTGGCTGACC
Ldb0655			AGGCCAGGTCATAGAATTGC
			GTAGCCCTTGATACCCGACA
Ldb0657	F	GCCGGTCTCTTTTCCACTCT	GGTCCGTTCAAAGCAGTCATAG
	R	GGTCCGTTCAAAGCAGTCATAG	
Ldb0658	F	CAGGCAAATAGCAGCGAGAT	CCATTCACCTTCCATATGCTC
	R	ATACGAAGCGGTGAACTTGC	GGAGCCGTCTTCGCCTTT
			CAGCAGGGTCTTGACAGTTG
Ldb0660	F	GATGATCTGGGGAATCATGC	
	R	GGCGATTAGGCTGTTCATGT	
Ldb0705	F	CCTGCAGAACTATGCACGTC	
	R	GGACTTCACCCGCAAAGATA	
Ldb0956	F	TCGTGTCAATCAACGGTGTTA	CTTTGGCTGGCCTTTAGTGT
	R	CTTTGGCTGGCCTTTAGTGT	TTGCCGGTGAAAACTTTGAT
			GTTGATGATCGGAGCGATG
Ldb1239	F	GTATTCCAGCCGATGGTGTT	TGACCTTATCGCCAACTTTCTT
	R	TGACCTTATCGCCAACTTTCTT	TTCGGTCATATCGTGATCCA
			ATTCCTTCTCGCCAACAACC
Ldb1245	F	GTCTGCTCTGGCCCTCAAGG	
	R	TCCGAACCGAGTCGTAAACCT	
Ldb1301	F	ATGATCGGAACCGACAAGAC	
	R	AGGCAAAACCGATGATTGTC	
Ldb1306	F	GTGACGTTGTTTCCATCGTG	CCGATCAAGGAGACGATGAA
	R	AAGAAGAGGCCGTTGGTGTA	AAGAAGAGGCCGTTGGTGTA
			CACGATGGAAACAACGTCAC
Ldb1663	F	CCGGTATCGTTGGCTCTTTA	TGCCTGCTTGCCTAAGAGTT
	R	TGCCTGCTTGCCTAAGAGTT	CGAAGACCAATAGAAAGTTGAACC
			AGGCCACGAATAAGACGATG
Ldb1810	F	GGACCTTGATTGCTGTCTGG	AAGACCGAGGGAACGAAGAA
	R	AAGACCGAGGGAACGAAGAA	ACAGCAATCAAGGTCCCAAT
			CCAGAAGATGAGTAAGGCGATG
Ldb1895	F	CGCCTGCAGACAGAGTTTTA	
	R	CCAGACTGGCACGTCATAGA	
Ldb2158	F	GGTGGCCTTCACTGGTCTTA	GGTAAATGGCACGGCGTAG
	R	GTTTTGGATTCTTCGGTTCG	GATCATCCATTCAGCCCAGTA
			CCTTCTTTCAAGTCCCCAAAC
Ldb2159	F	GTCCTCAGCCTCTTCACCAC	
	R	AAGACGAAGTAGAGGGCTTCAA	
Ldb2201	F	TTGATCTTGAGCTTGAACCAAA	
	R	CAGGCCAGAATCGTAAGACAC	

[a] F, forward; R, reverse.
[b] All primers in this group were reverse primers.

measured as described above. Cells from 1 ml of the second aliquot were harvested at $13,000 \times g$ and resuspended in 1 ml of MPL, adjusted to pH 5.25 with lactic acid, and incubated at 42°C for 90 min (acid-adapted sample). Then, acid tolerance was measured as described above. The acid adaptation factor was calculated as the survival of acid-adapted cells divided by the survival of non-adapted cells.

In order to monitor natural acid adaptation during growth, a single fresh colony was used to inoculate 1 ml of MPL and incubated for 8 h at 42°C without shaking. The culture was then diluted 1,000-fold in 25 ml of prewarmed MPL medium and incubated further overnight. The resulting culture (OD_{600} of <0.2) was diluted to an OD_{600} of 0.05 with prewarmed MPL. At 1-h intervals, the OD_{600} and pH were measured, and acid tolerance was determined as above.
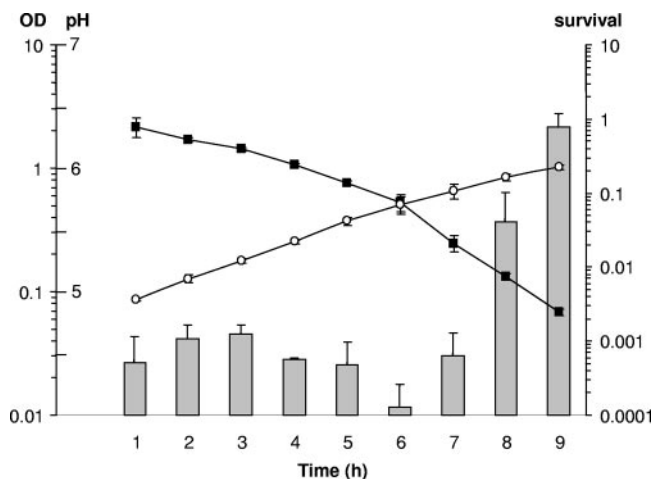
FIG. 1. Evolution of OD_{600}, pH, and acid tolerance during growth in MPL. Data represent the mean of three independent experiments. Standard deviations are indicated by error bars. ○, OD_{600}; ■, pH. The histogram indicates survival after acid challenge (pH 4.0).
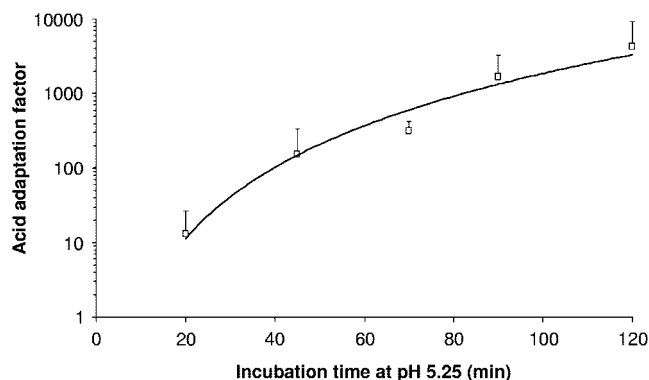

FIG. 2. Acid adaptation factor as a function of adaptation time. Acid tolerance (survival at pH 4.0) of an exponentially growing culture was measured after different periods of incubation at pH 5.25 and compared to acid tolerance of the nonadapted culture. Survival of the nonadapted culture varied between 8×10^{-6} and 2.6×10^{-4}, with a mean of 8×10^{-5}. Data represent the means for five independent experiments. Standard deviations are indicated by error bars.

A slightly modified protocol was used for acid adaptation experiments (see Table 4) in which cultures were grown in MPLm, with or without exposure to a sublethal pH of 4.75 in MPLm acidified with either HCl or lactic acid (adaptation), and tested for acid tolerance in MPLm acidified to a pH of 3.6 with HCl.

RNA extraction and cDNA preparation. For extraction of RNA, cells from 10 ml of culture with an OD_{600} of ~0.2, with or without acid adaptation treatment, were harvested at $2,600 \times g$ (10 min at 4°C). The pellet was resuspended in 100 μl of cold 10 mM Tris–1 mM EDTA buffer (pH 7.5), frozen in liquid nitrogen, and stored at −80°C. The cells were thawed on ice and broken by shaking with 0.6 g of glass beads (<106 μm in diameter; Sigma) (three times for 45 s each) in a Bio 101 Fastprep apparatus (Savant Instruments, Holbrook, NY) in the presence of 500 μl of acid phenol. One milliliter of Trizol (Life Technologies, Rockville, MD) was added to the aqueous phase and used to extract RNA as recommended by the supplier. DNA was removed using the DNA-free system (Ambion, Austin, TX) (0.04 U of DNase 1/μg of RNA; 30 min at 37°C). RNA was quantified by measurement of the OD_{260}. cDNA was obtained by reverse transcription of 5 μg of DNA-free RNA using Powerscript Reverse Transcriptase (Clontech Laboratories, Inc., Mountain View, CA) with 250 ng of random hexamers (New England Biolabs, Ipswich, MA) in a volume of 20 μl, as recommended by the supplier of the enzyme.

QPCR. Primers for quantitative PCR (QPCR) (Table 1) were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR was carried out in a 25-μl volume containing 10 μl of cDNA diluted 1,000-fold, specific primers (0.2 μM each), and 12.5 μl of ABsolute QPCR SYBR Green mix (ABgene, Epsom, United Kingdom). Thermocycling was performed using an ABI 7700 instrument (Applied Biosystems, Foster City, CA) using the following parameters: 1 cycle at 95°C for 15 min and 40 cycles at 94°C for 15 s and at 60°C for 1 min. Melting curve amplification was performed with temperature increments of 1.5°C per min in order to check that the amplification did not produce secondary products. PCR efficiency was checked for each primer set using serial dilutions of cDNA. Cycle threshold values were defined as the cycle number at which the fluorescence exceeded a fixed threshold value above the baseline. Induction of gene expression during acid adaptation, relative to gene expression before adaptation, was calculated using the comparative $\Delta\Delta C_T$ (where C_T is cycle threshold) method (30) with Ldb1245 as the internal control gene for normalization of the amount of cDNA in the reaction. The control gene Ldb1245 encodes a sigma A homologue and was chosen after verification that its expression level was not significantly affected by acid adaptation in the two independent physiological experiments: for a given amount of total RNA, the amount of Ldb1245 mRNA varied by a factor smaller than 1.4 between acid-adapted and nonadapted cells (data not shown). All QPCR measurements were performed in triplicate on samples derived from two independent cultures. The absence of genomic DNA was checked by QPCR using an aliquot of the DNA-free RNA samples before reverse transcription as templates and the Ldb1245 primers (Table 1).

Mapping of the starting points of transcription and motif search. Starting points of transcription were mapped using a 5′-3′ rapid amplification of cDNA ends kit (Roche Applied Science, Mannheim, Germany), as recommended by the supplier. Mapping was realized with RNA extracted from acid-adapted and nonadapted cells, using the primers listed in Table 1. Conserved sequence motifs were searched within the 500 bp upstream of the translation initiation codon using iMOMi (N. Pons, J. M. Batto, S. D. Ehrlich and P. Renault, submitted for publication), based on the MEME algorithm (7).

RESULTS

Evolution of acid tolerance during growth. During growth, LAB gradually acidify the culture medium and adapt to the lower pH. For *L. bulgaricus*, the extent of this adaptation during growth in a chemically defined medium (MPL) was evaluated by measuring the survival to an acid challenge (pH 4.0 for 30 min) (Fig. 1). During the first 7 h of growth, the pH of the culture gradually decreased from 6.4 to 5.4, and the survival after acid challenge fluctuated between 0.01% and 0.1%. Then, while the pH of the culture further decreased to 5.1 after 8 h and 4.8 after 9 h, the survival to acid challenge rapidly increased to 4% and 78%, respectively. These results clearly illustrate that during growth and acidification of the culture medium, *L. bulgaricus* acquires an improved tolerance to acid challenge. The onset of a rapid development of acid tolerance coincided with a medium pH between 5.4 and 5.1 and a culture OD_{600} between 0.65 and 0.85.

Experimental acid adaptation of *L. bulgaricus*. To dissociate the effect of adaptation to an acidic environment from potential growth-phase-related effects, we examined whether the exposure of an exponentially growing culture with an OD_{600} of 0.2 to a low but sublethal pH (5.25) could induce an improved survival to acid challenge (30 min at pH 4.0). The results presented in Fig. 2 show that such a response could indeed be induced and that the adaptation factor increased with the time of incubation at pH 5.25. After 90 min, the survival to acid challenge was 1,000-fold higher than in the nonadapted control sample.

Inventory of genes involved in acid tolerance and ion transporters. An analysis of the genome sequence of *L. bulgaricus*

TABLE 2. Genes involved in acid tolerance in LAB

Function and gene[a]	Description	Gene no. in *L. bulgaricus*[b]	Reference
Protein degradation			
clpP	ATP-dependent Clp protease proteolytic subunit	Ldb0624	28
htrA	HtrA-like serine protease	Ldb0140	16
DNA repair			
recA	Recombinase A	Ldb0599	41
uvrA	Excinuclease ABC, subunit A	Ldb0615	21
Cell envelope			
fabM	Fatty acid desaturase	NP	20
dagK	Diacylglycerol kinase	NP	58
ffh	Signal recognition particle protein Ffh	Ldb1372	27
dltC	D-Alanyl carrier protein	Ldb2146	10
Proton pumps and related functions			
atpABCDEFGH	H^+-transporting ATPase/ATP synthase	Ldb0705–Ldb0712[c]	26
gadCD	Glutamate decarboxylase	NP	47
adc	Aspartate decarboxylase	NP	1
hdc	Histidine decarboxylase	NP	35
odc	Ornithine decarboxylase	Ldb0547, Ldb1775[d]	6
mlf	Malolactic fermentation	NP	14
citCDEFG, citM, citP	Citrolactic fermentation	NP	31
Ammonia production			
arcABC	Arginine deiminase	NP	46
ureABCDEFG	Urease	NP	11

[a] Only functions that have been studied in detail, beyond an observation of induction in proteomic or transcription studies, are presented.
[b] NP, not present.
[c] Different subunits of the H^+-transporting ATPase are encoded by Ldb0705 to Ldb0712.
[d] The *L. bulgaricus* genome encodes two ornithine decarboxylases.

ATCC 11842 (52) reveals only few of the functions that have been implicated in acid tolerance in other LAB (Table 2). Of the enzymes that have been described to produce alkaline compounds, and thereby play a role in pH control, the genome only encodes two ornithine decarboxylases. *L. bulgaricus* does not possess (i) an arginine deiminase pathway, (ii) glutamate, histidine, or aspartate decarboxylase, or (iii) urease. Metabolic pathways for malolactic fermentation or citrolactic fermentation are also absent.

In contrast, of the H^+ and other ion transporters that have been described to play a role in acid adaptation, *L. bulgaricus* encodes an H^+ ATPase, a chloride channel, and a K^+ uptake permease (Table 3). An inventory of ion transporters encoded in the genome revealed 16 additional transporters, some of which could play a role in acid adaptation.

Induction of ion transporters during acid adaptation. In order to identify ion transporters involved in acid adaptation, *L. bulgaricus* was acid adapted using conditions based on the results described above (90 min at pH 5.25). RNA was extracted from adapted and nonadapted samples, and the relative expression of transporters was determined by reverse transcription followed by QPCR (RT-QPCR).

Transcripts were detected for all genes tested, in both adapted and nonadapted cells. The results presented in Table 3 show that among the 19 transporters tested, one (Ldb1239 protein) was induced 31- to 45-fold during adaptation, and three others were induced by a factor greater than 2 in two repetitions of the experiment with independent cultures. Re-

markably, these include the three CPX-type ATPases (Ldb1239, Ldb0660, and Ldb2158 proteins) present in the *L. bulgaricus* genome. CPX-type ATPases constitute a subclass of the P-type ATPases that are specialized in the transport of heavy-metal ions (5, 44, 49). The fourth gene is a cation:proton antiporter (Ldb0226).

The expression of six of the studied genes was repressed by a factor of about 2. These include a branched chain amino acid:proton symporter (Ldb0483 protein) and an Na^+/H^+ antiporter (Ldb1810 protein). Also repressed were a permease of unknown specificity (Ldb0162 protein), an ammonia transporter (Ldb1663 protein), a phosphate ABC transporter (Ldb0956 to Ldb0959 proteins), and a cation-transporting P-type ATPase (Ldb0456 protein). Neither the H^+-ATPase (Ldb0705 to Ldb0712 proteins) nor the K^+ uptake permease (Ldb0219 protein) were induced or repressed. Induction of the chloride channel-encoding gene (Ldb2201) was low (1.7-fold) but reproducible.

Transcription start site mapping and motif search. To determine whether a common regulator might control the different transporter genes, the 5′ ends of transcripts from the genes of which the expression varied by a factor of 2 or more were determined. Transcription start sites proved to be identical under both adapted and nonadapted conditions, indicating that the modulation of transcript levels did not rely on switching between two promoters. Start sites were determined for all genes but one (Ldb0956), and near-consensus sigma A promoter sequences were identified upstream of all

TABLE 3. Induction of ion transporters in *L. bulgaricus* ATCC 11842

Gene no. in *L. bulgaricus*[a]	Function	TC no.[b]	mRNA ratio[c]	
			Expt 1	Expt 2
Ldb1239	CPX-type ATPase; putative Cu transporter CopB	3.A.3.5.2	31.1	44.7
Ldb0660	CPX-type ATPase; putative Cu transporter CopA	3.A.3.5.1	2.1	3.5
Ldb2158	CPX-type ATPase	3.A.3.6.5	2.6	2.1
Ldb0226	Cation:proton antiporter	2.A.37.1.1	2.5	2.1
Ldb0341	Cation-transporting P-type ATPase	3.A.3.4.1	2.1	1.2
Ldb2201	Chloride channel	1.A.11.5.1	1.8	1.6
<u>Ldb0426</u>-Ldb0424	Putative cobalt ABC transporter	3.A.1.23.2	1.0	1.1
<u>Ldb0705</u>-Ldb0712	H+-transporting ATPase	3.A.2.1.1	1.1	0.8
Ldb0219	K+ uptake permease	2.A.72.1.1	0.8	1.0
Ldb2159	Na+/H+ antiporter	2.A.37.2.1	0.8	0.7
Ldb1301	Cation-transporting P-type ATPase	3.A.3.3.3	0.7	0.6
Ldb1895	Putative cation transporter	1.A.35.3.1	0.6	0.5
Ldb1306	Cation efflux protein; probable metal/H+ antiporter	2.A.4.1.1	0.5	0.6
Ldb0456	Cation-transporting P-type ATPase	3.A.3.2.4	0.5	0.5
Ldb1810	Na+/H+ antiporter	2.A.35.1.1	0.5	0.5
<u>Ldb0956</u>-Ldb0959	Phosphate ABC transporter	3.A.1.7.1	0.6	0.4
Ldb0483	Branched chain amino acid:H+ symporter	2.A.26.1.3	0.5	0.5
Ldb1663	Ammonia transporter	2.A.49.1.1	0.5	0.5
Ldb0162	Putative ion transporter	2.A.47.5.1	0.4	0.3

[a] For multicomponent transporters encoded by an operon, the first and the last genes of the operon are indicated, and genes of which the expression was measured are underlined.

[b] TC no., classification number of the nearest homologue in the Transport Classification Database.

[c] Amount of mRNA in acid-adapted cells/amount in nonadapted cells, measured by RT-QPCR; results for two independent experiments are presented. Each value represents the mean of three QPCR measurements.

these sites (Fig. 3). Ldb0660 appeared to be transcribed from two promoters, one directly upstream of the Ldb0658-Ldb0659-Ldb0660 operon and one situated further upstream, preceding Ldb0655 (Fig. 4A). The gene directly upstream of the Ldb0658-Ldb0659-Ldb0660 operon is a pseudogene (Ldb0656/Ldb0657), and it seems likely that in the course of the degradation of this gene, the accompanying transcription terminator has also been affected (Fig. 4A), resulting in readthrough of transcription. Ldb0655-Ldb0656/Ldb0657 transcription was not induced during acid adaptation (data not shown), but as a consequence of readthrough transcription from this operon, the apparent induction factor measured for the Ldb0658-Ldb0659-Ldb0660 operon will underestimate the induction of transcription initiation from the Ldb0658 promoter.

The promoter regions were subsequently analyzed with iMOMi (N. Pons, J. M. Batto, S. D. Ehrlich and P. Renault,

submitted) to identify shared motifs that could be indicative for coordinated regulation by a common regulator. A common motif, YKACARWYRTAARC comprising an internal inverted repeat (Fig. 4B and 5A), was only found in one or two copies in the promoter regions upstream of the CPX ATPase genes Ldb1239, Ldb0660, and Ldb2158. This motif was not found elsewhere in the *L. bulgaricus* genome. Nearly identical motifs were found in the promoter regions of homologues of Ldb1239 and Ldb0660, but not Ldb2158, in a number of other closely and more distantly related LAB (Fig. 5B).

Characterization of CPX-type ATPases. While the implication of several of the induced or repressed transporters in pH control is very conceivable, we focused on the CPX-type ATPases for further analysis because (i) all three intact ATPases of this type in the genome were induced, (ii) one of these was very strongly induced, (iii) a common sequence motif could be identified in the promoter regions of these genes, and
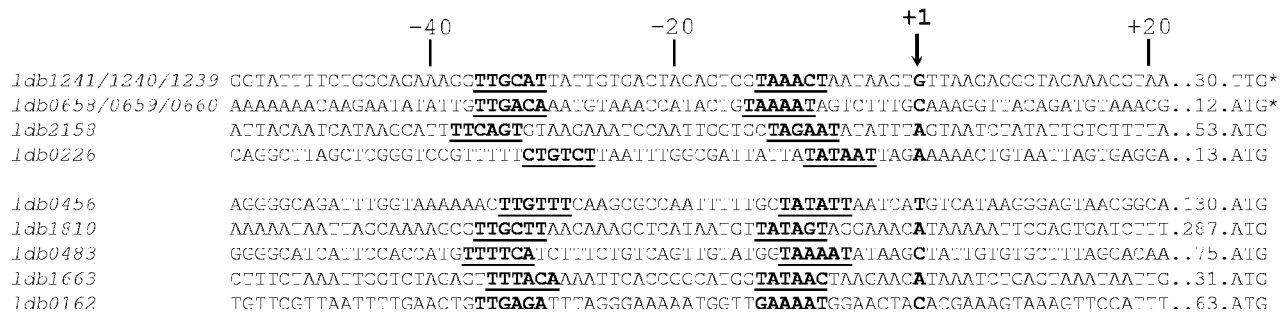

FIG. 3. Transcription start sites. Transcription start sites (+1) and putative −35 and −10 promoter sequences (underlined) are presented for a selection of ion transport genes. The number of nucleotides between the presented sequence and the translation start codon shown in the right part of the figure is indicated. Putative ribosome binding sites were identified upstream of the indicated start codons (data not shown). Genes Ldb1239 and Ldb0660 (∗) make up part of the operons. In this case, the transcription start site of the operon is presented and the distance to the start codon of the first gene in the operon.
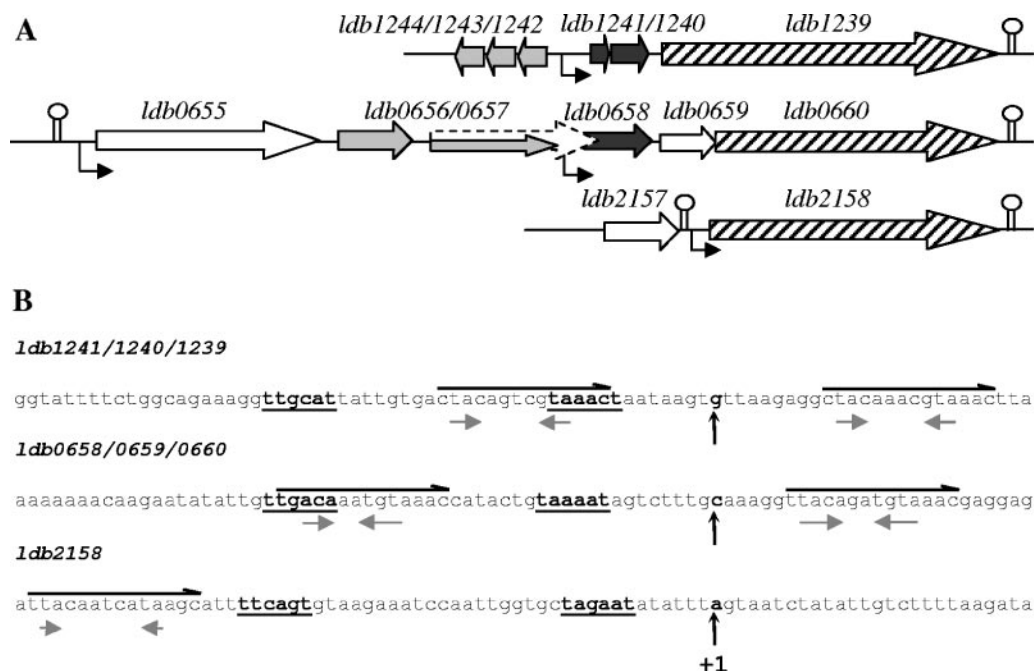
FIG. 4. Genetic organization of CPX-ATPase loci. (A) CPX-ATPase coding genes are indicated by hatched arrows; intact (Ldb0658) or fragmented (Ldb1241/Ldb1240) copY-like regulator genes are indicated by black arrows. Transcription start sites are indicated by thin black arrows. Putative transcription terminators are indicated by stem-and-loop structures. Ldb0655 encodes a putative hydrolase, Ldb0659 encodes a conserved hypothetical protein, and Ldb2157 encodes a conserved hypothetical protein. Ldb1242/Ldb1243/Ldb1244 constitutes a 6-phospho-beta-glucosidase pseudogene, and Ldb0656/Ldb0657 constitutes a penicillin binding protein (pbp) pseudogene. For Ldb0657, the gray arrow indicates the part that is homologous to other pbp genes. At the site where these homologues contain a stop codon, the Ldb0657 open reading frame continues (dotted arrow), probably because gene degradation removed the stop codon. Likewise, gene degradation may have degraded a transcriptional terminator that may have been present downstream of the original pbp gene. (B) Promoter regions upstream of the Ldb1241/Ldb1240-Ldb1239 and Ldb0658-Ldb0659-Ldb0660 operons and the Ldb2158 gene. Transcription start sites are indicated as +1. Putative −35 and −10 promoter sequences are underlined. The common sequence motif detected by iMOMi is indicated by black arrows, and the inverted repeats within this motif are indicated by gray arrows. The motif is repeated twice in the promoter regions of the Ldb1241/Ldb1240-Ldb1239 and Ldb0658-Ldb0659-Ldb0660 operons.

(iv) as described below, at least two of these proteins probably make up part of a coordinated system.

The Ldb1239 and Ldb0660 proteins are homologous to the copper-transporting CopB and CopA proteins of *Enterococcus hirae* (36), respectively. The alignment of these proteins clearly illustrates the conservation of the TGE, DKTGT, and GDGI NDAP motifs common to P-type ATPases and the CPX motif of the subgroup of heavy-metal-transporting CPX-type ATPases (49) (Fig. 6). The conserved ALGLA membrane channel motif directly following the CPX motif strongly suggests that the Ldb1239 and Ldb0660 proteins are involved in the transport of monovalent Cu or Ag ions (49, 51). Of these, Cu seems the most relevant as this metal is present in low concentrations in milk (42). The CPXALGLA motif is only partially conserved in the Ldb2158 protein. Overall protein homology and a shared sequence motif in the promoter region, which may indicate a common regulation mechanism, suggest that this protein, too, may be involved in copper transport, although transport of another heavy metal ion cannot be excluded.

Proteins Ldb0660 and Ldb2158 lack the N-terminal domain that is present in most other heavy metal ATPases (Fig. 6). This difference is not considered to be important, however, as a similar lack has been observed in homologous proteins from

several other bacteria, and in *E. coli* ZntA this domain was shown not to be essential for function or specificity (34).

Role of CPX-type ATPases during acid adaptation. These observations raise the question of the significance of the induction of CPX-type ATPases and, more precisely, a putative copper homeostasis control system during acid adaptation. As copper is essentially known for its function as a cofactor in redox enzymes, one may wonder whether the slightly different history of acid-adapted and nonadapted cells in terms of aeration may be responsible for the induction of the transporters. For acid adaptation, cells were subjected to centrifugation and resuspended in fresh medium (with a lower pH), while nonadapted cells were frozen immediately after centrifugation (see Materials and Methods). Thus, in a control experiment in which nonadapted cells were resuspended in fresh medium (without pH change) and incubated for 90 min at 42°C, expression of CPX-type ATPases was not induced (data not shown), thereby eliminating this explanation.

A second explanation could be that lactate, used to acidify the adaptation medium, binds copper ions (49), thereby changing the effective copper concentration and inducing the system. To test this possibility, acid adaptation experiments were repeated using a medium the pH of which had been adjusted with HCl instead of lactic acid. The results shown in Table 4

A

```
CTACAGTCGTAAAC
CTACAAACGTAAAC
TGACAAATGTAAAC
TTACAGATGTAAAC
TTACAATCATAAGC
```
ykACArwyrTAArC *
 TACAnnTGTA **

B

CopB homologues

ldb1239 *	CT**ACA**GTCG**TA**AAC-N_{17}-CT**ACA**AACG**TA**AAC
Lpl *	CT**ACA**ATTG**TA**AAC-N_{12}-TT**ACA**GATG**TA**GAC
Lla	TT**ACA**ATTG**TA**AAC
Sth	TT**ACA**GATG**TA**AAC

CopA homologues

ldb0660 *	TG**ACA**AATG**TA**AAC-N_{27}-TT**ACA**GATG**TA**AAC
Lpl **	TG**ACA**ATTG**TA**AAC-N_{25}-TT**ACA**AGTG**TA**AAC
Lac *	TT**ACA**AATG**TA**AAT-N_{13}-TT**ACA**TTTG**TA**AAC
Ljo *	TG**ACA**AGTG**TA**AAC-N_{12}-TT**ACA**TTTG**TA**AAC
Lla *	TT**ACA**CGTG**TA**AAC
Sth *	CT**ACA**AATG**TA**AAC

ldb2158	TT**ACA**ATCA**TA**AGC

FIG. 5. Alignment of the putative CopY binding sites. (A) Alignment of the five motifs shown in panel B. *, consensus motif; **, CopY binding site in gram-positive bacteria (39). (B) Alignment of the motifs here with motifs found in promoter regions of homologous genes in a number of other LAB. *Lpl*, *Lactobacillus plantarum* WCFS1 (24); *Lac*, *Lactobacillus acidophilus* NCFM (3); *Ljo*, *Lactobacillus johnsonii* NCC 533 (40), *Lla*, *Lactococcus lactis* IL-1403 (9); *Sth*, *Streptococcus thermophilus* LMG18311 (8). *L. acidophilus* and *L. johnsonii* do not contain CopB homologues. In the other bacteria mentioned, *copA* and *copB* are part of different transcription units and are transcribed separately or cotranscribed with a *copY* homologue (*) or with a homologue of Ldb0659 (**). In the latter two cases, the motifs shown are found upstream of the *copY* or Ldb0659 homologue, respectively. All bacteria mentioned except for *S. thermophilus* contain an Ldb2158 homologue, but the conserved motif is found only in the *L. bulgaricus* promoter region.

demonstrate that expression of the CPX-type ATPases was induced under these conditions, thereby eliminating the possibility of a lactate effect and emphasizing the role of the pH of the medium.

Finally, adaptation experiments were performed using a medium without added copper. While MPLm contains 0.01 μM CuSO$_4$, in this experiment *L. bulgaricus* was cultured, and adaptation was performed in MPLm without this medium component. The results presented in Table 4 show that this omission did not affect the induction of the CPX-type ATPases during acid adaptation.

DISCUSSION

Acid adaptation is a complex process that can mobilize a large spectrum of different cellular functions. As currently viewed, the mobilized functions include those that control intracellular pH and proton motive force to prevent the deleterious effects of acidification and those that repair damage to proteins and DNA once it has occurred.

A survey of the *L. bulgaricus* genome sequence revealed that this bacterium possesses few of the proteins that have been implicated in the first class of functions in other bacteria. We therefore focused on the potential role of proton and ion transport functions other than the H$^+$-ATPase, which has previously been studied in detail in other LAB (15, 22, 25, 37) and which is also present in *L. bulgaricus*. Transporters likely involved in acid adaptation were revealed by measuring their induction or repression in response to a pH shift. Although modification of gene expression under these conditions is not a prerequisite for a transporter to be involved in adaptation, this approach allowed the identification of a number of trans-

porters with a potential role in this process. These include several proton symporters and antiporters and an ammonia transporter.

Surprisingly, the results of this study showed that acidification of the culture medium also, and significantly, induced the enhanced expression of putative heavy-metal-transporting CPX-type ATPases. Proteins Ldb0660 and Ldb1239 appeared homologous to CopA and CopB from *E. hirae*, responsible for the import and export, respectively, of Cu$^+$ (48, 57). Copper is both important as a cofactor in redox enzymes and very toxic, necessitating a tight regulation of its intracellular concentration (49). In *E. hirae*, the *copA* and *copB* genes are part of one operon which also encodes a chaperone (CopZ) and a repressor (CopY) and is believed to ensure copper homeostasis. CopY represses the expression of the operon by binding to the operator sequence TACANNTGTA (56). If copper is in excess, it will bind to CopY, thereby preventing the binding of CopY to the operator and inducing expression of the operon (39, 50). A shortage of copper also induces the expression of the operon, but the mechanism of this induction is not understood (36, 56). Paradoxically, either condition thus induces both the importer CopA and the exporter CopB, a feature which has been hypothesized to act as a safety mechanism to avoid copper intoxication in the case of sudden variations in ambient copper availability (49).

The CopA and CopB homologues in *L. bulgaricus* share essential sequence motifs with the proteins from *E. hirae* and are part of the same phylogenetic group of transporters, two features that strongly suggest that the *L. bulgaricus* proteins, too, are involved in copper transport (44, 49, 51). The presence of a CopY homologue (Ldb0658 protein) in *L. bulgaricus*,
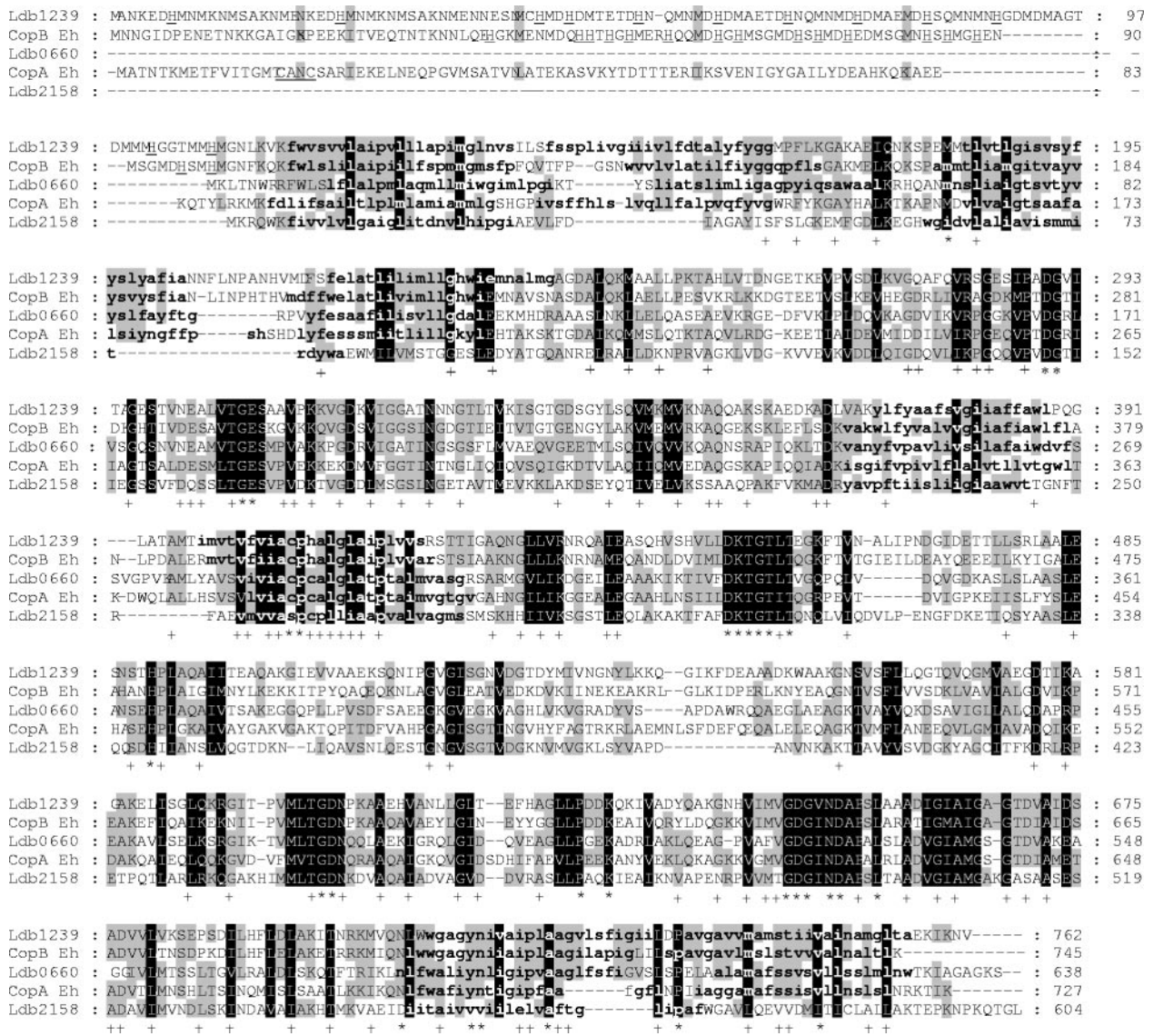
FIG. 6. Alignment of *L. bulgaricus* CPX-ATPases and Cu-ATPases from *E. hirae*. Residues conserved or similar in all sequences are shaded black; those conserved in at least three sequences are shaded gray. Residues putatively involved in metal binding are underlined (CXXC motif or histidine-rich region). Transmembrane domains detected by TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) are indicated in bold, lowercase letters. For the shaded residues, the following symbols apply: +, residue conserved in at least 70% of the probable copper-transporting ATPases of the P-type ATPase database (http://www.patbase.kvl.dk/); ∗, residues conserved in all of the probable copper-transporting ATPases of the P-type ATPase database.

TABLE 4. Effect of different adaptation media on induction of CPX-type ATPases

Gene no. in *L. bulgaricus*	Induction factor under the indicated conditions[a]			
	Lactic acid		HCl	
	+Cu	−Cu	+Cu	−Cu
Ldb1239	32.7	34.6	20.5	21.2
Ldb0660	14.5	9.0	3.3	2.3
Ldb2158	5.7	4.5	4.2	3.9

[a] Values represent the induction factor (relative gene expression [acid-adapted/nonadapted]), measured by RT-QPCR for the conditions indicated. Induction factors for lactic acid cannot be compared to values in Table 3 because of differences in medium composition (MPLm was used instead of MPL; see Materials and Methods) and pH of adaptation. +Cu, MPLm (containing 0.01 μM CuSO₄); −Cu, MPLm from which CuSO₄ was omitted. Absolute values of induction factors obtained with HCl and with lactic acid cannot be compared because these conditions represent qualitatively and quantitatively different stress levels for the cell.

encoded by the gene upstream of and in the operon with the *copA* homologue Ldb0660, and the strong resemblance between the conserved motif upstream of the CPX-type ATPase genes in *L. bulgaricus* and the *E. hirae* CopY binding site reinforce this hypothesis. The CopY homologue contains in its C-terminal part a CXCX₃C motif, slightly different from the CXCX₄CXC motif present in CopY of *E. hirae* thought to be the copper binding site (44a). Further support comes from the presence of a *copY* pseudogene upstream of the *copB* homologue Ldb1239. A protein resembling CopZ (Ldb0480 protein) is encoded elsewhere in the genome, and although *L. bulgaricus* lacks an electron transport system, several proteins that exhibit a copper binding motif were detected (data not shown).

In *L. bulgaricus*, Ldb0660 and Ldb1239 are situated in dif-

ferent transcription units which would theoretically allow a fine-tuning between import and export functions. On the other hand, the CopA and Ldb0660 protein orthologue from *E. coli* has been described as an exporter rather than an importer (17, 43); we can therefore not exclude the possibility that in *L. bulgaricus* both the Ldb1239 and Ldb0660 proteins are involved in copper export.

The results presented in this study strongly suggest that acidification of the culture medium affects copper homeostasis in *L. bulgaricus* and thereby threatens cell viability. Induction of the three CPX ATPase genes was observed after acidification with HCl as well as with lactic acid. The mechanism underlying this induction is as yet not clear, however. The strong resemblance of the ATPases, the CopY homologue, and the putative operator sites of the three separate transcription units to the respective elements of the intensively studied *E. hirae* system suggests that CopY plays a key role in this induction. It is not clear if and how copper could play a role in the induction, because this also takes place in a culture medium that contains only trace amounts of copper, if any at all. It is tempting to believe that CopY operator fixation is also sensitive to stimuli other than copper concentration, which is directly related to medium acidity.

Transport activity of the *E. hirae* CopB protein is strongly pH dependent, and its efficiency rapidly decreases below pH 6 (48). If this property is shared by the *L. bulgaricus* Cop homologues, an increased expression at low pH values could serve to compensate reduced efficiency and ensure copper homeostasis. This system could thus be involved in avoiding indirect damage as a consequence of medium acidification, which could have dramatic effects for the cell if not properly addressed.

Several recent studies in other bacteria evaluated the changes in gene expression during growth or short time exposure to acidic conditions using a genome-wide transcriptomic approach (*Lactobacillus plantarum* [38], *Helicobacter pylori* [4, 33, 55], *Mycobacterium tuberculosis* [18], and *Streptococcus pneumoniae* [32]). Interestingly, one of these studies identified CopA as strongly induced in the highly acid-resistant stomach colonizing bacterium *H. pylori* after 48 h of growth at pH 5.5 (compared to a control grown at pH 7.2) (4).

Transcriptomic and proteomic (29) analyses of acid adaptation rapidly reveal the induction or repression of a multitude of new genes, in addition to those classically implicated. Some of these changes are expected or can be understood intuitively (e.g., modifications of metabolic activity), while others are more surprising but not necessarily less important. They allow us to further develop our understanding of the profound and pleiotropic effect that acidification of the culture medium, brought about by LAB, has on the physiology of the bacterial cell.

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